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NON-MOTOR SYMPTOMS IN PARKINSON'S DISEASE: MODELING AND MECHANISMS

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Non-motor symptoms in Parkinson's disease: modeling and mechanisms

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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"Here, the images of a laboratory mouse and a scientist are combined, because they are connected to each other."

Andrei Kharkevich

ABSTRACT

Parkinson's disease (PD) is a common neurodegenerative disorder, typically characterized by the progressive death of midbrain dopaminergic neurons projecting from the substantia nigra to other areas within the basal ganglia. Historically, PD has been diagnosed as a purely motor disorder dominated by bradykinesia (slowness of movement), rigidity, resting tremor, and postural instability. While there are no approved disease-modifying therapies, these symptoms can be counteracted by dopamine replacement therapies based on the use of L-Dopa and dopamine receptor agonists.

In the past decades, the classic view of PD as an exclusively motor disease has been challenged by increasing evidence showing that patients display a wide range of non-motor symptoms (NMS), including hyposmia, sleep disturbances, cognitive impairment, depression and anxiety. These ailments often appear in the early, pre-motor stage of the disease and progressively worsen, significantly affecting the patient's quality of life. The neuropathological mechanisms underlying NMS are still poorly understood, but clinical findings have consistently shown the involvement of both the dopaminergic and non-dopaminergic systems. Interestingly, these symptoms only partially respond to dopaminergic treatments used to handle motor deficits, complicating the pharmacological management of patients. The increasing demand for more effective therapies for NMS indicates the importance of developing translational approaches based on the use of appropriate animal models.

In this thesis, we developed and validated a mouse model of PD for the study of NMS. This model is characterized by a bilateral partial degeneration of the dopaminergic system achieved through intra-striatal injection of 6-hydroxydopamine (6-OHDA). We found that this model presents only subtle gait modifications reminiscent of early stage PD. Most importantly, we showed that the 6-OHDA lesion impairs olfactory discrimination, disrupts circadian rhythm, causes long-term memory deficits, as well as depression- and anxiety-like behaviors. Using a combination of anatomical and pharmacological approaches, we validated this model for the study of NMS and described the effects produced on these deficits by administration of dopaminergic and non-dopaminergic drugs.

Altogether, these studies provide a well-characterized tool for the study of NMS in PD, as well as information on molecular and neural targets implicated, thereby opening new vistas for the design of broader therapeutic interventions.

LIST OF SCIENTIFIC PAPERS

- I. Bonito-Oliva A, **Masini D**, Fisone G. (2014)
A mouse model of non-motor symptoms in Parkinson's disease: focus on pharmacological interventions targeting affective dysfunctions.
Frontiers in Behavioral Neurosciences 8:290
- II. **Masini D**, Lopes-Aguiar C, Bonito-Oliva A, Papadia D, Andersson R, Fisahn A, Fisone G. (2017)
The histamine H3 receptor antagonist thioperamide rescues circadian rhythm and memory function in experimental parkinsonism.
Translational Psychiatry 7(4):e1088
- III. **Masini D**, Bonito-Oliva A, Bertho M, Fisone G. (2018)
Inhibition of mTORC1 signaling reverts cognitive and affective deficits in a mouse model of Parkinson's disease.
Frontiers in Neurology 9:208

Other publications not included in this thesis:

Rivetti di Val Cervo P, Romanov RA, Spigolon G, **Masini D**, Martín ME, Toledo EM, Giole M, Feyder M, Pifl C, Yi-Han N, Saez SP, Linnarsson S, Wernig M, Harkany T, Fisone G, Arenas E. (2017)
Induction of functional dopamine neurons from human astrocytes in vitro and mouse astrocytes in a Parkinson's disease model.
Nature Biotechnology 35(5) 444-452

Caggiano V*, Leiras R*, Goñi-Erro H, **Masini D**, Bellardita C, Bouvier J, Caldeira V, Fisone G, Kiehn O. (2018)
Midbrain circuits that set locomotor speed and gait selection.
Nature 553(7689) 455-460

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LIST OF ABBREVIATIONS

4E-BP	Eukaryotic initiation factor 4E-binding protein
6-OHDA	6-hydroxydopamine
BLA	Basolateral nucleus of the amygdala
CeA	Central nucleus of the amygdala
D1	Dopamine receptor 1
D2	Dopamine receptor 2
D3	Dopamine receptor 3
DA	Dopamine or Dopaminergic
EC	Enhanced care
EPM	Elevated plus maze
ERK	Extracellular signal-regulated kinase
FST	Forced swim test (porsolt)
GPe	Globus pallidus external part
GPI	Globus pallidus internal part
H3R	Histamine receptor 3
L-Dopa	L-3,4-dihydroxyphenylalanine
MAO-B	Monoamine oxidase B
MOBgl	Main olfactory bulb glomerular layer
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mTORC	Mammalian target of rapamycin
NA	Noradrenaline or Noradrenergic
NMDA	N-methyl-D-aspartate
NMS	Non-motor symptoms
NOR	Novel object recognition
OB	Olfactory bulb
OF	Open field
PD	Parkinson's disease
S6K	Ribosomal protein S6 kinase
SC	Standard care
SN	Substantia nigra
SNC	Substantia nigra pars compacta
SNr	Substantia nigra pars reticulata
STR	Striatum dorsal part
TH	Tyrosine hydroxylase
TST	Tail suspension test
VMAT2	Vesicular monoamine transporter 2
VTA	Ventral tegmental area

1 INTRODUCTION

1.1 THE EXPANDING SYMPTOMATOLOGY OF PARKINSON'S DISEASE

Parkinson's disease (PD) is a complex neurological disorder classically characterized by the dopaminergic denervation of the basal ganglia, due to the death of dopamine-producing cells in the substantia nigra (SN). The resulting dopamine deficiency leads to the parkinsonian motor syndrome described as poverty and slowness of movement paralleled by increased muscle tone. In the clinical practice, those cardinal symptoms are referred to as bradykinesia and muscular rigidity, and are usually accompanied by tremor at rest and postural instability.

Although PD diagnostic criteria are classically focused on motor dysfunctions, the conceptualization of the disease continuously evolved since Dr. James Parkinson's first detailed description two centuries ago¹. Currently, the symptomatology of the disease is recognized as heterogeneous, involving neurotransmitters other than dopamine and regions of the nervous system outside the basal ganglia. Moreover, it is now well accepted that the disease has a slow and progressive nature, which begins decades before diagnosis can be made and manifests itself with a broad range of deficits including non-motor symptoms (NMS)².

NMS comprise autonomic dysfunctions such as cardiovascular, urogenital and gastrointestinal manifestations, sleep disorders with dream enactment behavior and daytime somnolence, sensory disorders such as hyposmia and chronic pain, cognitive impairment and neuropsychiatric conditions including depression and anxiety. Importantly, NMS can precede the typical parkinsonian motor syndrome, which develops only after approximately 70% of the SN dopaminergic cells are lost³.

PD is often viewed as a synonym to motor parkinsonism and its diagnostic criteria consider most NMS as an early sign of disease onset, medically referred to as a prodrome⁴. Consequently, NMS are often under-recognized since patients remain unaware that some of their behaviors may be related to PD, embarrassed to discuss NMS that are not prompted by the doctor and even lack time for disclosure when attention is focused on motoric criteria⁵. As a result, clinicians often fail to recognize NMS during consultations⁶.

Recently, the International Parkinson and Movement Disorders Society presented a new guideline for PD diagnosis, with the aim of updating clinical practice and standardizing the process across centers. Amongst the changes, dementia was removed⁷ as an exclusion criterion for PD. In parallel, they proposed a set of criteria for the diagnosis of prodromal PD which, as for now, is only intended for research. These changes highlight a much-needed expansion of the disease concept, and the prodromal PD criteria have raised an interesting discussion among scientists regarding its feasibility⁸⁻¹⁰. Given the advances and controversies, it is likely that the upcoming years will see an interesting debate concerning non-motor features of PD.

Clinical and experimental research on the prodromal phase have important implications, not only for the identification of early pathogenic events, but also for optimizing the success of future therapeutic strategies, as well as developing treatments against non-motor features which represent a significant clinical burden.

1.2 DOPAMINE REPLACEMENT THERAPY: A 'GOLEM' IN NEUROSCIENCE

With disease-modifying treatments remaining elusive, the management of PD symptoms is often achieved through dopaminergic replacement therapies that aim at increasing dopamine availability or directly stimulating dopamine receptors. In this context, administration of the dopamine precursor L-Dopa (L-3,4-dihydroxyphenylalanine), is the gold standard treatment approach (in fact, L-Dopa responsiveness is part of the diagnostic criteria).

L-Dopa effect in PD patients was initially demonstrated in 1961. This first successful clinical trial was preceded by 3 major scientific breakthroughs, all done in a matter of five years: dopamine was shown to be present in the brain, to be enriched in the mammalian basal ganglia and to be dramatically reduced in the brain of parkinsonian patients. As a result of these seminal articles, the research field was rapidly pushed forward (see history^{11,12}) causing an increase in topic-related publications in the years that followed (see blue bars in *Figure 1*).

Since then, we have gathered an enormous amount of information concerning the pathophysiology of this disease. Indeed, we have learnt that the disease develops from a complicated interplay between genetics and environment, with humans being exclusively affected by a time-dependent cascade of events ultimately leading to lysosomal dysfunction and α -synuclein aggregation¹³. Moreover, we learnt that L-Dopa affords symptomatic relief for a subset of motor deficits, that it does not modify the progressive neuronal degeneration and that prolonged administration of this drug results in the emergence of a range of undesirable side-effects¹⁴. Despite these limits, to this day, L-Dopa remains the most efficacious treatment for the symptomatic control of PD and all other conventional approaches still rely on the basic concept of dopaminergic replacement strategies one way or another. In fact, in addition to L-Dopa, the current PD pharmacotherapy includes the use of dopamine agonists to directly activate dopamine receptors, monoamine oxidase B (MAO-B) and catechol-O-methyl transferase inhibitors to prevent dopamine metabolic degradation and anticholinergic agents to correct the imbalance between dopamine and acetylcholine.

Fast forward from 1961 to 2018 and here we remain, the dopaminergic replacement therapy is likened to a Golem, a creature from mythology, powerful yet potentially dangerous, a helpful creature that may yet run amok at any given moment. We cannot stop disease progression, yet PD symptoms are mostly under the Golem's control.

In fact, thanks to dopamine replacement therapies, motor parkinsonism no longer overshadows the non-motor symptomatology, as indicated by a large body of evidence

suggesting that the global burden of NMS in PD has a greater impact on life quality than motor symptoms^{15–18}.

Braak stages of PD¹⁹

Perspective Box

The pathological aggregation of α -synuclein into toxic fibrils and deposits is a hallmark of the disease, being accompanied and followed by neuronal degeneration in PD. According to the current concept, PD is a synucleinopathy that propagates in an ascending pattern via directly connected neuronal pathways. Based on these progressive events, the course of the pathology can be divided into *stages*, as proposed by Braak and colleagues. Initially, the PD pathology is only detected in the olfactory bulb and in two basal cranial nerves nuclei, *i.e.*, the glossopharyngeal and the vagal nerve (stage 1). This corresponds to the development of hyposmia and autonomic dysfunctions, including obstipation. Successively, the pathology propagates into the pontine areas of the locus coeruleus, the raphe nuclei, and the reticular formation (stage 2). This brainstem affection may cause REM sleep disorder, which is one of the most specific predictor of PD. The following stage is characterized by the involvement of the SN and the anterior olfactory nucleus (stage 3), whereas significant rates of degenerating neurons in the SN pars compacta are only seen later on (stage 4). Motor symptoms only emerge at stage 4 or later.

This view has been challenged by some²⁰.

The broad range of NMS and their complex pathophysiology will be discussed in detail in individual sections within this thesis. Importantly, a subset of NMS is refractory to L-Dopa treatment²¹, while others may even worsen due to dopamine replacement therapies. For instance, the increase of dopamine synthesis produced by L-Dopa treatment has been shown to reduce serotonin levels in the PD brain^{22,23} and serotonergic disruption plays a prominent role in the expression of neuropsychiatric symptoms occurring at the onset of the disease²⁴ (note that serotonergic neurons are capable of decarboxylating L-Dopa into dopamine and then releasing it. This process is cytotoxic to serotonergic cells due to oxidative mechanisms²⁵).

The expanding symptomatology of PD has triggered a growing interest in clinical and preclinical research on NMS and highlighted the need to develop and validate new preclinical models (see red bars in *fig. I*). The focus of this thesis is to bridge the gap between researchers and clinicians by optimizing an animal model for the study of non-motor symptoms in Parkinson's disease.

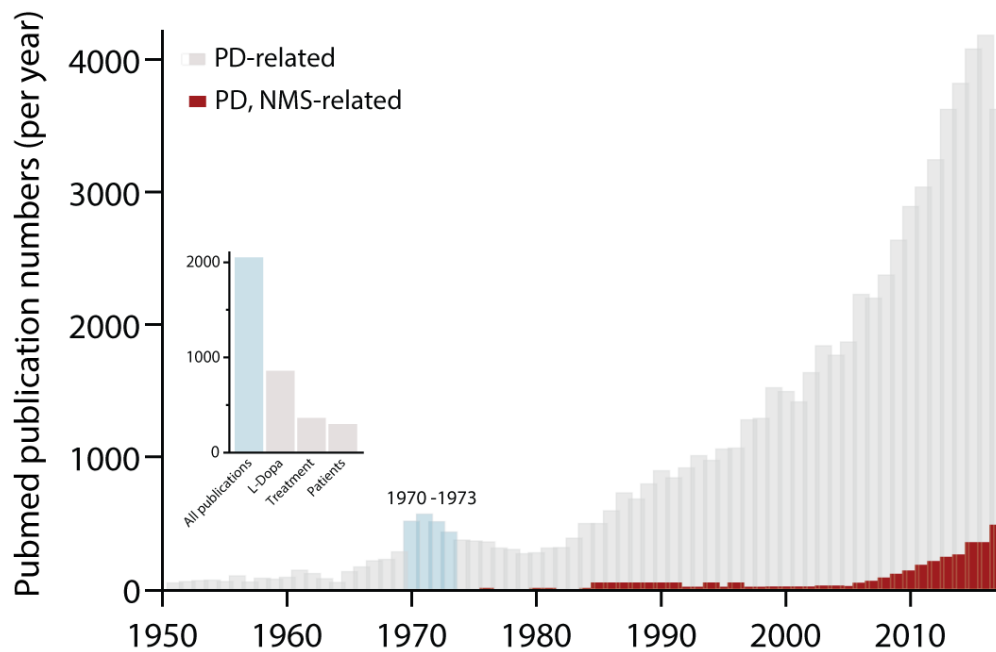


Figure 1. Diagram showing the total number of PD-related publications (grey bars) as compared to the number of NMS-related publications (red bars). Note the spike in PD publications between 1970-1973 (light blue bars), following the discovery of L-Dopa as a therapy for PD patients. During that period, the most frequent words on article titles were “L-Dopa”, “treatment” and “patients”, in order (inlet). Currently, the number of publications on NMS related topics is still low in comparison to the total number of PD related publications. Data acquired from PubMed, adjusted following the MeSH Database and examined using open-ended ‘free text’ analyses on JMP statistical software.

1.3 REQUIREMENTS FOR PRECLINICAL RESEARCH

An animal model should fulfill a set of criteria to be considered relevant for the study of the corresponding human pathology. *Predictive validity* relies on pharmacological correlation, where the treatment known to be effective in humans should improve symptoms in animals. In other words, it corresponds to the ability of the model to respond to the treatments, as expected based on human data. *Face validity* refers to the degree of behavioral and anatomical similarity between the model and the disorder that is modeled. Finally, *construct validity* regards the accuracy with which the model measures what it is intended to measure. In other words, it indicates the correspondence between symptoms and underlying neuronal mechanisms in both human and animal species. Together, these validity checks ensure the predictive power of an animal model²⁶.

It is important to note that the use of animal models in research requires a deep knowledge of their intrinsic limitations. Within the frame of this thesis, one must be aware that PD does not occur spontaneously in animals other than humans. However, despite the human-specific nature of PD, the characteristic features of the disease can be mimicked in animals (rodents and non-human primates) through administration of various neurotoxins and drugs that disturb dopaminergic neurotransmission²⁷ or alternatively by genetic approaches²⁸. The understanding that preclinical models are an incomplete representation of the human

condition and that the modeling approach is contingent to the scientific questions that it aims to address, is a fundamental prerequisite for translational research (see reviews^{29–31}).

In the specific context of NMS, it has to be considered that the occurrence of these symptoms in animals is studied through behavioral tests that, by definition, require a relatively intact motor repertoire. For this reason, an appropriate animal model of NMS should not display the drastic motor components of PD.

1.3.1 Ethical considerations

Ethical considerations are an important aspect when choosing an adequate preclinical model. First, no responsible scientist wants to use animals or cause them unnecessary suffering if it can be avoided, be it physical or emotional stress. Therefore, the use of animals in research should comply with the ethical framework established by governmental institutions, animal welfare groups, veterinaries and experts in ethics. Second, living systems are extremely complex and as for now, we lack alternatives to study interrelated processes occurring in the central nervous system.

1.4 THE USE OF 6-OHDA

With the nigrostriatal degeneration being recognized as the primary pathogenic factor in PD³², the path was paved for the search of animal models. Interestingly, shortly after the discovery of the L-Dopa effect, the 6-OHDA (6-hydroxydopamine) neurotoxin was described^{33,34}.

6-OHDA is a structural analogue of dopamine and noradrenaline, and exerts its toxic effects on catecholaminergic neurons by inducing mitochondrial oxidative stress and ultimately cell death³⁵. Due to its inability to cross the blood-brain barrier, 6-OHDA has to be injected directly into the brain (via stereotaxic surgery). Ungerstedt³⁶ pioneered the use of 6-OHDA to produce the PD phenotype by injecting the toxin bilaterally into the SN of the rat, obtaining an animal model with impaired voluntary movements and high mortality rate. Following this study, several protocols of 6-OHDA administration have been published and this neurotoxin has become a widely used approach for modeling parkinsonism in rodents.

The popularity of the 6-OHDA model lies on its ability to mimic the dramatic loss of dopaminergic neurons in PD. The resulting dopaminergic depletion remains stable for several months, thereby simplifying the experimental evaluation (particularities are on section 1.5). Moreover, 6-OHDA can be used to model distinct stages of the human pathology, simply by varying the localization and extent of the lesion.

However, as every experimental model, the 6-OHDA-induced PD model has its limitations. The toxin has an acute effect and selectively kills catecholaminergic cells, consequently, it does not replicate the full spectrum of clinical PD features. For instance, the degeneration of cholinergic neurons³⁷ is not fully replicated and the serotonergic system is only partially

affected³⁸. In addition, although 6-OHDA causes dopaminergic depletion, it fails to develop other pathological hallmarks of the disease, such as the appearance of Lewy bodies containing α -synuclein aggregates³⁹.

Despite those limitations, the 6-OHDA approach represents one of the best and most widely used PD rodent models and has been an essential tool for testing a variety of anti-parkinsonian treatments over the years.

1.5 THE BILATERAL PARTIAL 6-OHDA MODEL OF PARKINSON'S DISEASE

In the preclinical practice, the striatal dopaminergic depletion can be induced by infusing 6-OHDA in one of three different brain areas, the dopaminergic cell bodies in the **substantia nigra** (SN), the **medial forebrain bundle** connecting those cells to their targeted regions, and the **striatum**, which is the most prominent release site for dopamine in the basal ganglia. Researchers can perform either unilateral or bilateral lesions. Unilateral lesions are mostly used for the study of motor parkinsonism, whereas bilateral lesions are the appropriate choice for the study of NMS (discussed on section 1.6). Importantly, bilateral lesions that incur total dopaminergic depletion are not compatible with life.

The **substantia nigra** is a relatively small structure. It has a thin profile, being elongated on the rostro-caudal direction and is neighbored by the ventral tegmental area. Anatomical descriptions of the disease course showed that in early PD the ventral tier is more affected than the dorsal tier^{3,40}. Moreover, injections of 6-OHDA in the medial SN result in larger cell loss^{41,42}. Taken together, these anatomical factors make it difficult to generate a partial depletion of dopamine by targeting the SN (see structure in red on *fig.2*).

SN partial lesion with 6-OHDA and sprouting

Perspective Box

There is evidence in the literature indicating that ‘medium-size’ lesions done in the SN (< 70% of dopaminergic loss) are accompanied by re-innervation of the striatum, via axonal sprouting⁴³⁻⁴⁷ (also the effect of drug treatment in PD models⁴⁸ and evidence in patients⁴⁹) with consequent motor improvement⁴⁷ (measured as rotational bias in unilaterally lesioned rodents). Interestingly, the process is described as being driven by new axons entering the striatum rather than sprouting of remaining axons from spared cells⁵⁰. Thereafter, long-term studies must always report lesion size and stability.

The **medial forebrain bundle** is a dense neural pathway that contains, amongst others, the ascending fibers of dopaminergic cells projecting from the SN to striatal areas. The axons of dopaminergic cells are variculated and contain the dopamine transporter, which accounts for the uptake of 6-OHDA into the cells. Due to its anatomical connections, this area is the most frequent injection site used to generate unilateral total lesions, yet researchers have shown that dose-dependent loss, although technically difficult, can also be accomplished⁵¹⁻⁵³.

Whereas, the direct injection of 6-OHDA into the SN or medial forebrain bundle yields a rapid death of dopaminergic cell bodies, followed by degeneration of striatal terminals, toxin injections into the **striatum** produce a slower degradation of nigrostriatal fibers through a retrograde mechanism that occurs over a period of weeks^{54–57}. Time-course studies with total unilateral striatal lesion in rodents reported that the initial damage to the dopaminergic axons occurs within 24 hours, with the extent of the lesion-site peaking at the third week. In parallel a retrograde process is taking place, causing a loss of dopaminergic cell bodies that peaks within 28 days (rat⁵⁴ and mice⁵⁸). At this final stage, the lesion remains stable for a prolonged period of time^{55–57}.

Compared to SN and medial forebrain bundle, the administration of 6-OHDA into the striatum is easier to perform, due to its relatively larger size and dorsal position. These anatomical features allow for a better control of the lesion extent, critical when the goal is to achieve a partial degeneration. Importantly, this area can be reliably targeted inducing NMS in the absence of severe motor impairment (discussed below) (see structure in blue on *fig.2*), well mimicking the initial dopaminergic deficiency observed in the dorsolateral striatum of PD patients⁵⁹.

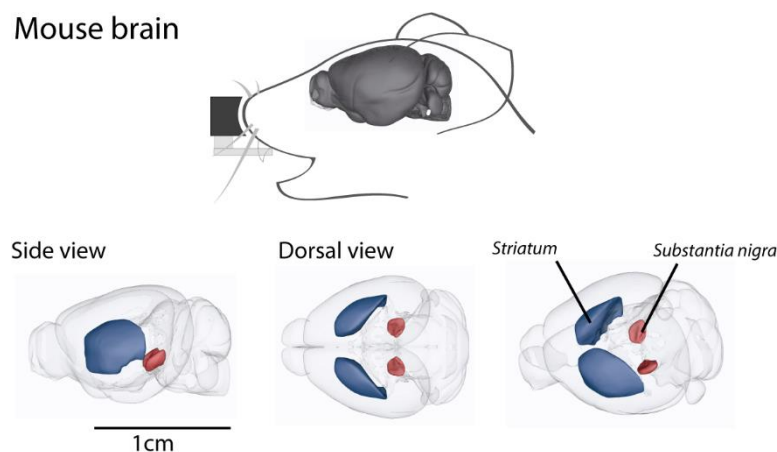


Figure 2. Schematic mouse brain depicting the anatomical location of the striatum and substantia nigra, two areas which are commonly targeted by 6-OHDA administration. Note how the striatum, in blue, is a much larger structure allowing for easier access and reproducibility of partial lesions (scale bar as indicated).

Regardless of the injection site, the extent of the lesion can be manipulated by changing the toxin concentration and volume of injection, variables that differ between mice and rats. It has also been shown that both young and aged male rats are more susceptible to 6-OHDA insult compared to females (SN, unilateral lesions)⁶⁰. Subsequently, the researcher must consider all above-mentioned factors in order to reliably generate a partial lesion model of NMS in PD.

For an adequate modeling of NMS in PD, the use of bilateral rather than unilateral dopaminergic lesion is needed to avoid compensatory effects between lesioned and non-

lesioned hemispheres⁶¹. Moreover, the lesion must be partial to preserve motor repertoire and allow an appropriate evaluation of behavioral performance.

To our knowledge, the first study that used a bilateral 6-OHDA lesion for the sole purpose of modeling NMS in PD was published in 2005 by Ferro⁴¹ and colleagues. In this work, the toxin was injected at high concentrations in the substantia nigra of rats, resulting in high levels of dopaminergic depletion with cell death in both the SN and the ventral tegmental area. Moreover, the protocol of injection resulted in high mortality rates, with almost half of animals being lost within the first 20 days. The high mortality rate in 6-OHDA lesioned animals had already been reported previously^{62–65} and is thought to be due to reduced feeding motivation (*i.e.*, adipsia and aphagia), consequent dehydration and weight loss^{62,66}. Hence, Ferro et al., were able to test only the less severely lesioned rats (~80% dopaminergic depletion) and described the presence of cognitive deficits (as assessed in the water maze task). However, the protocol induced a significant impairment in spontaneous locomotion, a confounding factor that limited their behavioral profiling.

In 2008, Tadaiesky⁶⁷ et al. carried out the first comprehensive study where rats were bilaterally injected in the dorsal striatum to achieve a 60% decrease in the number of dopaminergic cells within the substantia nigra and 40% decrease in the density of striatal tyrosine hydroxylase (TH, the rate-limiting enzyme of catecholamine synthesis). Following the lesion, rats showed depressive-like behaviors, anhedonia and behavioral despair, as indicated by diminished sucrose consumption and increased immobility in the forced swim test. Furthermore, lesioned rats had increased anxiety as determined by reduced entries to the open arms of the elevated plus maze. Finally, lesioned rats needed more time to find the platform in the water maze task and failed to display social odor recognition despite having normal olfactory function. These features indicate a deficit in cognitive function. Importantly, in this model the described NMS occurred in the absence of changes in spontaneous locomotion, as measured with the open field test.

Up to now, rats are the species of choice to model NMS with 6-OHDA partial lesions. This is not only a legacy of the first experiments but also a result of the overall reduced survival observed when bilateral lesions are performed in mice. Both adipsia and aphagia were reported since the start of 6-OHDA use in the central nervous system⁶⁸ and while rats can be force fed, mice cannot withstand such approach. In spite of these limitations, the use of mice to study NMS is often preferable, since this species offers more options in combination with transgenic approaches, giving access to a full range of modern research tools.

In conclusion, partial lesions of the nigrostriatal dopaminergic system are considered to be analogous to the early stages of human Parkinson's disease and can be induced reliably by striatal injection of 6-OHDA. Technically, the main challenge is to find the correct 6-OHDA concentration needed to achieve NMS without confounding motor effects and to reduce post-operative mortality. Importantly, the generation of reliable mouse models of NMS would foster further investigation by reducing costs and opening a door to the use of a large array of genetically modified animals as the field develops.

1.6 A MOUSE MODEL TO STUDY NON-MOTOR SYMPTOMS IN PD: INITIAL RESULTS

In this thesis, we studied NMS using a mouse model initially developed in our research group by Bonito-Oliva and colleagues⁶⁹. This model was generated by a bilateral injection of 6-OHDA in the dorsolateral striatum (4 µg/µl; 1 µl/site), resulting in a 70% striatal reduction of dopaminergic fibers and 80% decrease in hippocampal TH immunoreactivity. The bilateral lesion had no effect on spontaneous motor activity but was accompanied by cognitive deficit, as assessed by behavioral and electrophysiological experiments.

The hippocampus is involved in cognitive function and receives both dopaminergic and noradrenergic input. Noradrenergic innervation originates mainly from the locus coeruleus whereas dopaminergic fibers originate from the ventral tegmental area (VTA) and substantia nigra pars compacta (SNc)⁷⁰, both important for hippocampus-dependent learning and memory⁷¹⁻⁷³. Due to 6-OHDA-induced toxicity, and in line with the above-mentioned results, TH expressing cells in the substantia nigra and locus coeruleus dropped by 65% and 25%, respectively, prompting the authors to perform a series of experiments in order to investigate the relative role of these two neurotransmitters in cognitive performance. For this aim they pretreated lesioned mice with desipramine, a blocker of the noradrenaline transporter, which protects noradrenergic fibers from the toxin. Their results showed that the cognitive deficit was still present even after desipramine pretreatment, indicating the unique role of dopamine in this memory task.

Follow-up experiments showed that the cognitive deficit was paralleled by reduced synaptic plasticity in the dentate gyrus, a region of the hippocampus that plays a key role in learning⁷⁴. The deficit in synaptic plasticity was counteracted by activation of hippocampal dopamine D1 receptors (through administration of L-Dopa and SKF81297, a selective D1 receptor agonist⁷⁵) while D2 / D3 receptor activation (through administration of pramipexole) resulted ineffective. Finally, synaptic plasticity was dependent on intact extracellular signal-regulated kinase (ERK) signaling. Importantly, ERK signaling pathways are implicated in gene expression including immediate early genes, which are in turn involved in various forms of synaptic plasticity^{76,77}.

In summary, the initial article described a new mouse model for the study of cognitive deficits in PD and showed that dopaminergic depletion, though only partial, is sufficient to cause cognitive impairment, which is particularly dependent on D1 receptor activation and ERK signaling in the hippocampus.

The basal ganglia are subcortical structures that coordinate vital behaviors including movement. The striatum receives the majority of the basal ganglia input, and projects to the output nuclei via two pathways that work concomitantly to modulate behavior. D1 receptor expressing striatal medium spiny neurons project to the substantia nigra pars reticulata, whereas, D2 receptor medium spiny neurons project to the globus pallidus pars externa. Classical functional models of the basal ganglia propose that the direct and indirect pathways exert opposing effects on motor behavior; specifically, activation of the *direct pathway increases locomotor activity* (via D1-expressing cells), whereas the *indirect pathway suppresses motor activity* by exerting a tonic inhibitory tone (via D2-expressing cells). This model posits that any weakening of the *direct pathway*, or any strengthening of the *indirect pathway*, will result in poverty and slowness of movement which are cardinal features in PD⁷⁸.

Dopamine D1 receptor expressing cells and the antiparkinsonian effect

L-Dopa's antiparkinsonian effect occurs upon its first dose and is mediated by activating dopaminergic receptors. However, precisely which dopaminergic receptor type is primarily responsible for L-Dopa's effect is usually not defined in the literature^{79–81}. The clinical use of D2 receptor agonists, such as pramipexole, but not D1 agonists^{82,83} to treat parkinsonism may suggest that D2 receptors in the *indirect pathway* are the primary mediator of the therapeutic effects of L-Dopa. However, experimental studies have demonstrated a critical role for the D1 receptor-expressing medium spiny neurons, originating from the *direct pathway*, to the promotion of motor activity^{84–87}. Therefore, D1 receptors are crucial for the motoric recovery observed in PD patients after L-Dopa treatment.

2 AIMS

The overall goal of this thesis was to investigate the behavioral effects of a bilateral partial striatal 6-OHDA lesion, and to optimize a reliable method for the preclinical study of NMS in PD.

In order to achieve this objective, our **first aim** was to perform a series of behavioral tests and anatomical analyses to provide *face validity*, by showing the degree of similarity between the proposed model and the NMS features observed in PD patients.

Upon identification of appropriate behavioral correlates, our **second aim** was to assess *predictive validity* by showing that pharmacological treatments known to be effective in patients improved symptoms in our mouse model.

Finally, in the **third aim** we provided *construct validity* by appropriately selecting protocols that identify the correspondence between symptoms and the underlying neuronal mechanisms based on literature regarding both clinical data and other pre-clinical models.

3 RESULTS AND DISCUSSION

3.1 INCREASING SURVIVAL OF CATECHOLAMINE-DEPLETED RODENTS

(manuscript in preparation)

As previously discussed, catecholaminergic lesions induced by 6-OHDA administration are often associated with lower rates of survival, mainly caused by adipsia and aphagia^{88–90} and have been described as strikingly similar to the lateral hypothalamic syndrome (read more⁹¹). Animal loss due to lesioning varies between 20% and 50% amongst articles and in many cases, due to conflict of interest, is not clearly reported.

On the other hand, the brain is incredibly resilient to insult and low extent lesions often lack clear behavioral output. In between the system's resilience and a model with measurable NMS, lies the success of a NMS model of PD. For instance, genetic animal models of PD with progressive dopamine degeneration, usually start to show NMS once cell loss achieves the 70% range. However, because of the progressive nature of these models, cells continue to die, motor impairment becomes salient and ultimately animals are sacrificed due to humane end point (for example see⁹²).

Throughout this project, we have conceptualized and then introduced a series of steps to aid recovery, reduce post-operative stress and ultimately increase survival of 6-OHDA lesioned mice. All procedures were approved by the local ethical committee (Stockholm Norra Djuretiska nämnd) and followed the EU directive (2010/63/EU). During the last 4 years, we kept a detailed description of each mouse used in our experiments, including general information regarding health status and perioperative care from one week pre-surgery to three weeks post-surgery.

The entire project (including all trial experiments, 4 published articles and three papers in preparation) employed 996 mice. Approximately half of the animals were treated with the standard approved protocol, while for the other half, an 'Enhanced Care (EC) protocol' was applied. Survival curves for both groups were compared with the Kaplan-Meier method^{93–95}, a statistical approach common to clinical trials, and our results show that the EC protocol increases survival rates, thereby reducing the number of mice needed in a project (*fig.3*). Moreover, the EC protocol includes a series of steps that respect the prerequisite of simple infrastructure and low associated costs. The details regarding the EC protocol are part of a manuscript in preparation, the manuscript describes the perioperative care and contains a series of statistical analyses that allowed us to examine which factors were mainly predictive of survival.

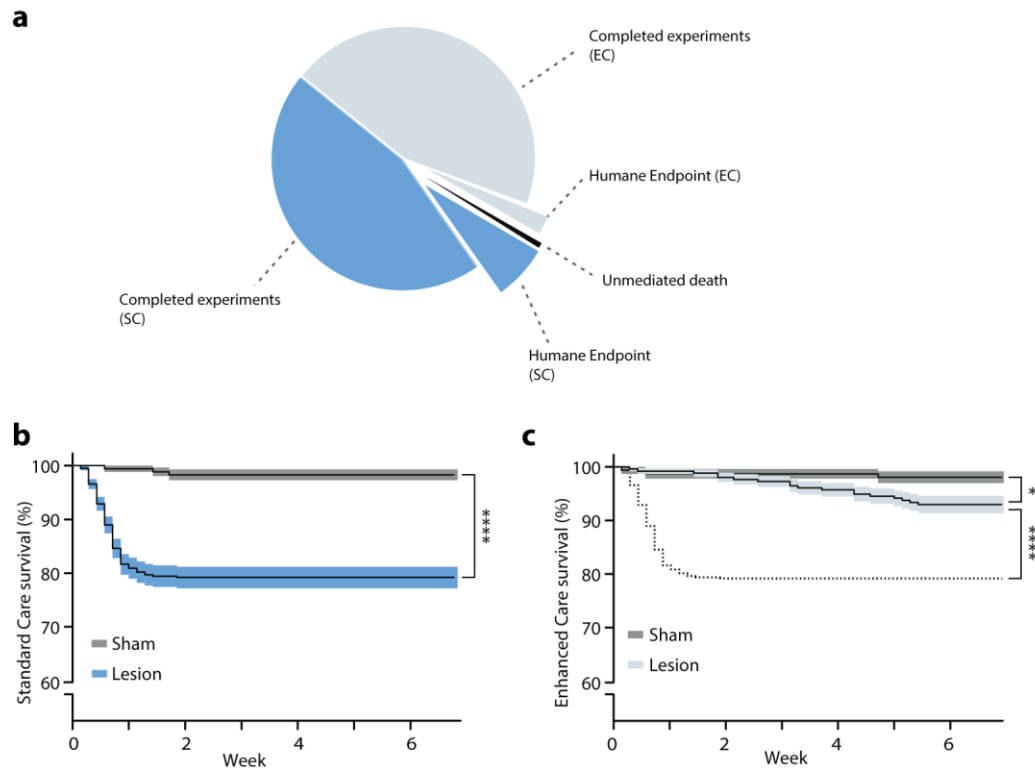


Figure 3. Tailored perioperative care enhances survival of mice with depletion of the catecholaminergic system. **(a)** Pie chart showing the percentage of mice that underwent either the Standard Care (SC) or the Enhanced Care (EC) protocol. For each group, mice that completed the experiments, reached humane end point or were lost during the recovery period (*i.e.*, unmediated death) are graphed. **(b)** Survival curves of control (Sham, $n = 176$) and Lesion ($n = 409$) mice treated according to the SC protocol following bilateral partial lesion of the dorsolateral striatum with 6-OHDA. Matel-Cox test (Logrank analyses) showed higher mortality risk for the Lesion group (**** $p < 0.0001$) compared to Sham. **(c)** Survival curves of Sham ($n = 155$) and Lesion ($n = 256$) mice treated according to EC protocol. Note the improved survival rate of Lesion mice. Matel-Cox test (Logrank analyses) indicates that Lesion mice are still at higher hazard risk (Sham vs Lesion, * $p = 0.025$). Yet, comparison of the survival rates between Lesion groups treated with SC (dashed line) and EC (light blue) show a significant improvement (**** $p < 0.0001$). The proposed protocol reduces mortality from 20% to 5%. Graphs in b and c show survival percentages plotted with the Kaplan-Meier method, with survival curves represented by lines and SEM plotted as asymmetrical shadow zones (no subjects were censored). Graphpad prism software.

3.2 BRAIN WIDE EFFECTS OF STRIATAL 6-OHDA LESION

(manuscript in preparation)

To better understand which brain areas are affected by the intrastriatal injection of 6-OHDA, we performed a brain wide analysis of TH immunoreactivity in lesion and sham operated mice, followed by a comparison of expression levels. This project contains the reconstruction of a whole-brain map for the 6-OHDA induced TH depletion and allowed us to pin-point affected areas and identify a variety of new brain regions that hold interest for the study on NMS.

Dopamine is produced in the cytosol by the TH-mediated enzymatic conversion of L-tyrosine into L-Dopa, which in turn, is converted into dopamine by the aromatic L-amino acid decarboxylase^{96,97}. While dopaminergic cells are mainly located in the SN and VTA, dopamine can be further converted into noradrenaline (NA) in other brain regions (*i.e.*, locus coeruleus). Therefore, the use of conventional TH immuno-histochemical methods⁹⁸ is inadequate to the purpose of discerning between catecholaminergic⁹⁹ cells (*i.e.*, DA and NA). Thus, anatomical distribution of catecholaminergic neurons and their targeted areas becomes a key element in the interpretation of any results based on TH staining.

This anatomical consideration is particularly relevant in the context of this thesis. As previously discussed, 6-OHDA administration has a generalized effect on both dopaminergic and noradrenergic cells, and although dopaminergic specificity can be achieved through desipramine pretreatment, we purposely decided not to pre-treat animals as noradrenergic deficit may underline a variety of PD-related NMS. For instance, noradrenaline in the basal forebrain promotes wakefulness, in the amygdala it is involved in response to stressful stimuli, and in the thalamus it modulates the sensation of pain^{100–104} (for review¹⁰³).

The method used to map TH immunoreactivity relies on a holistic approach by co-registering full coronal section images, taken on an epifluorescence microscope, to the Allen mouse brain atlas. It allowed us to gather information on more than 170 regions within the mouse brain ($n = 5/\text{group}$ for a total of 312 coronal sections). With this approach, we were able to confirm the lesioning effect initially observed in the SN, hippocampus and locus coeruleus. Moreover, we detected a variety of affected areas not previously described (*fig.4*).

Although the main target of the dopaminergic fibers originating in the substantia nigra pars compacta (SNc) is the striatum (STR dorsal part), a portion of these fibers targets the **globus pallidus** (GP)¹⁰⁶. With fibers arborizing profusely in the GPi (internal part) and more sparsely the GPe (external part)^{107–110}. Accordingly, lesions to the SNc in both rodents and monkeys cause a reduction in dopamine levels within the GP and a relatable reduction in TH immunoreactivity^{111–113}. Some of these fibers pass through the pallidum *en route* to the striatum, and innervate both regions. These fibers are likely affected by our protocol. Still, retrograde and anterograde labeling studies have shown that some dopaminergic fibers arise as a nigropallidal projection separately from the nigrostriatal projection^{114–117}. Nigropallidal fibers are conceivably not damaged by our lesion protocol.

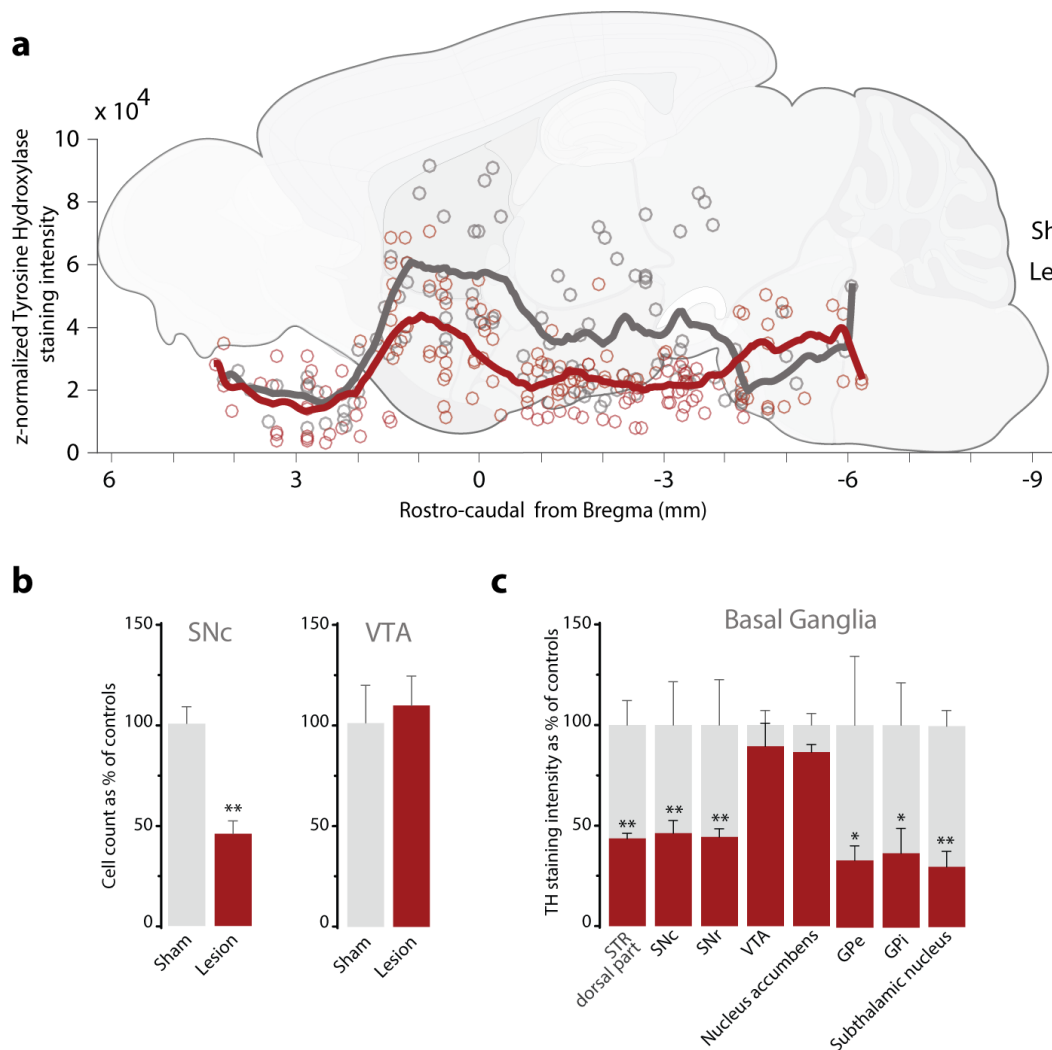


Figure 4. Lesion mice were perfused and their brains dissected for immunohistochemistry. Coronal sections throughout the brain were cut at the vibratome and stained for Tyrosine hydroxylase. Images were co-registered to the Allen mouse brain atlas and staining was quantified via a Matlab custom made script. **(a)** Line graph shows the normalized z-score staining values from rostral-caudal direction (fit line), in Sham and Lesion mice, where circles represent specific sample values. The graph was superimposed to a sagittal image of the mouse brain to aid interpretation. **(b)** Bar graph shows the 6-OHDA induced cell loss in Lesion mice, expressed as cell counts (% of control mice) for the substantia nigra pars compacta (SNc, left) and the neighboring ventral tegmental area (VTA, right). **(c)** Bar graph shows the catecholaminergic loss in Lesion mice expressed as TH staining intensity (% of control mice) within basal ganglia regions [(striatum (STR) dorsal part, substantia nigra pars reticulata (SNr), globus pallidus external and internal part (GPe, GPi)]. Mann-Whitney test, * $p < 0.05$, ** $p < 0.01$; $n = 5/\text{group}$. Data presented as mean \pm SEM. Nomenclature and acronyms follow Allen atlas specifications¹⁰⁵.

The **subthalamic nucleus** receives sparse collaterals from the SNc, as shown by studies in rats¹¹⁸, monkeys¹¹⁶ and humans¹¹⁹. Though characterized by low density of DA axons, it has been shown that the nigrosubthalamic pathway can directly modulate the activity of subthalamic neurons and that this pathway is subject to degeneration in MPTP-treated monkeys¹¹², and in our mouse model.

The dendrites of SNc dopaminergic neurons radiate ventrolaterally into the **substantia nigra pars reticulata** (SNr). Dopaminergic release in this area differs from the other basal ganglia nuclei because it is dendritic, rather than axonal^{120–122}. It has been shown that the interaction of dendritically released DA with somatodendritic autoreceptors regulates DA cell activity and may also affect SNr neurons (those are GABAergic). However, SNr neurons lack postsynaptic DA receptors, making it unclear how locally released DA modulates their activity.

Notably, significant reductions of dopamine in the globus pallidus, subthalamic nucleus and SNr have been reported in *postmortem* studies on brain tissue from PD patients¹²³ and in our mouse model a reduction in TH immunoreactivity indicates a similar phenotype.

The **nucleus accumbens** is located in the ventral striatal formation and receives dopaminergic projections from the ventral tegmental area (VTA). This pathway is directly involved in reinforcing and addictive responses that initiate drug addiction^{124–127} (for review¹²⁸). Interestingly, recent studies have highlighted the occurrence, in PD patients, of behaviors that develop as non-motor side effects of prolonged dopamine replacement therapy (*i.e.*, addiction and perseverative features). In our PD model, we initially did not observe differences in TH immunoreactivity between sham and lesioned accumbal areas (as shown in *fig.4c*). However, a detailed analysis of **shell** and **core**¹²⁹ sub regions, highlighted that there is a reduction in TH fibers restricted to the shell but not the core of the nucleus accumbens (*data not shown*). Dopamine release in the shell and the core region serve distinct functions, with the shell mediating the association between sensory stimulus and unpredicted rewards, while the core works as an interface between motivation and action (for more¹²⁵). Further studies in our research group aim at understanding possible implications of dopamine replacement therapy on the development of addictive-like behaviors.

The abovementioned examples show how an anatomically resolved lesion map can potentially uncover Parkinson disease-relevant structures and offer researchers a practical framework for future investigations. Importantly, the method used here has the holistic approach usually associated to high technological demands, yet our method relies on the use of common epifluorescence microscopes and a Matlab script to co-register the images with the Allen atlas. Though it increases workload, due to the necessity of individually co-registering images, the process can be applied with low technical demand.

3.3 MOTOR DOMAIN CONTROL EXPERIMENTS (Paper I)

As discussed above, the PD symptomatology involves both motor and non-motor symptoms. The ability to model one or the other of these classes of deficits is challenged by the significant, although not complete, overlap between the brain areas and mechanisms that underlie them. On the other hand, the progressive nature of the disease and the differential onset of these symptoms suggest that NMS can be modeled in animals by inducing only a partial neuronal degeneration. In preclinical models of PD, a correlation between the magnitude of cell loss in the substantia nigra and the degree by which spontaneous movements are affected has been observed (see for instance¹³⁰). As previously mentioned, motor impairment in preclinical research represents a potential hinder for the study of NMS, which relies on behavioral tests where the ability of the mice to move must be for the most part intact.

Based on these considerations and in order to validate our NMS model, we carefully examined the effects produced by our 6-OHDA lesion on a number of motor paradigms. Our results showed that the partial bilateral lesion of the striatum does not affect spontaneous locomotion, measured in the novel home cage test⁶⁹ and the open field test^{131,132}. Successively, we have also run a variety of tests specific to the motor domain, which are known for their higher sensitivity, such as gait analyses¹³¹, cylinder test⁶⁹, grip strength and rotarod (*data not published*).

In PD patients, spontaneous movements, particularly those of automatic nature, show slowness in execution. For instance, patients can present reduced speed (*i.e.*, hypokinesia), when initiating and executing a single movement and progressive reduction of its amplitude, up to complete cessation^{133–136}. These features are common in parkinsonism because the basal ganglia are involved in the performance of automatic movements such as gait⁸⁵. **Gait** is affected by several hypokinetic features including reduced stride length and decreased off-ground elevation of the feet, leading to short stepping and shuffling, which are associated with reduced arm swinging and predominance of flexor posture⁵⁹.

A comprehensive gait analysis using ventral plane videography showed that partially lesioned mice had normal stride length and frequency but presented subtle impairments of gait dynamics affecting specifically the hindlimbs (*table I & fig. 1, Paper I*).

Next, we tested the effect of the lesion on motor learning and motor coordination by using the **rotarod** test, where the mice are placed on a circular rod rotating at a fixed or increasing speed and the latency to fall is measured. While the fixed-speed paradigm is generally used to assess motor learning, the accelerating protocol is considered a better approach to measure motor coordination (know-how^{137–140}) (*fig. 5*). In short, on day 1 the animals were familiarized with the apparatus during three consecutive trials at a fixed speed of 4 rpm (*i.e.*, rotation per minute). Upon 2 h resting interval, mice were returned to the rod and latency to fall was measured at different fixed-speed values with increasing task difficulty. On day 2 mice were again tested on a fixed-speed protocol with higher rpm range. Two-way RM ANOVA

showed no interaction between groups and speed throughout the test ($F_{9,126} = 1.84$, $p = 0.07$), indicative of no motor learning deficits (*fig.5b*). On day 3, mice were tested with the acceleration protocol, in which the rod speed increased from 4 to 38 rpm within 5 min. In this test, Lesion mice showed lower latency to fall when compared to controls ($p < 0.0001$, Student's t-test, two-tailed). The parsimonious explanation of why Lesion mice only fail on the acceleration version of the rotarod test is that subtle alterations in gait dynamics affect coordination and cause animals to fall. This effect is only observable when the task requires constant adaptation to changes in speed.

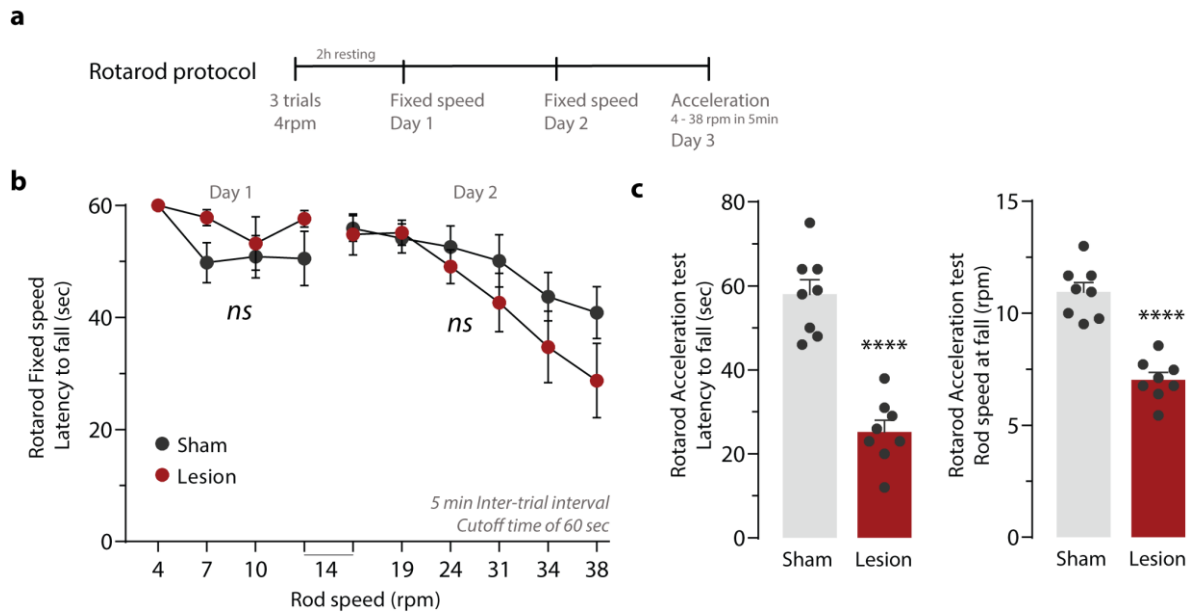


Figure 5. Comparison between two rotarod test protocols used to assess motor performance in mice. **(a)** Schematic experimental timeline. Animals were first trained on the rotarod apparatus in three consecutive trials with a fixed speed of 4 rpm. Upon a 2 h resting interval mice were tested at different fixed speeds (4, 7, 10 and 14 rpm). On day 2 mice were tested again at 14 rpm and then at a higher range of speeds (19, 24, 31, 34 and 38 rpm). On day 3 mice were tested according to the acceleration protocol, where the rod speed progressively increased from 4 up to 38 rpm within 5 min. **(b)** Line graph showing latency to fall (sec) during the fixed speed test on day 1 and 2 for each speed tested, in Sham and Lesion mice. **(c)** Bar graphs showing performance during the accelerating version of the rotarod test measured as latency to fall (sec, left panel) and speed with which it occurred (rpm, right panel). Two-tailed Student's t-test; **** $p < 0.0001$; $n = 8/\text{group}$. Data presented as mean \pm SEM.

We previously reported that 6-OHDA lesioned mice have a significant reduction in the total number of rearing events as assessed with the cylinder test⁶⁹. This data was later corroborated by counting rearing events during the open field test (*data not shown*). The observed reduction in rearing was therefore detected in two different apparatuses and interpreted as an example of forelimb hypokinesia. In order to exclude the possibility that such hypokinesia was due to muscle weakness, also present in PD patients¹⁴¹, we performed the **grip strength** test¹⁴². Our results showed that Lesion and Sham mice performed similarly on the grip

strength (3 trials avg, $p = 0.80$; max strength, $p = 0.91$, Student's t-test, two-tailed, $n = 12/\text{group}$) leading us to exclude muscular weakness, a feature associated to higher disease severity in PD patients¹⁴¹.

In conclusion, the model employed in this study reproduces an initial stage of symptomatic PD, with no effect on spontaneous locomotion. We believe that the subtle alterations in gait dynamics do not represent a potential hinder to the study of NMS. We did observe a reduction in vertical movements (rearing), in absence of muscle weakness. To compensate for this particular deficit, we modified some of our behavioral tests. For instance, we lowered the position of the odor source during olfactory discrimination tests (see below).

3.4 OLFACTORY DYSFUNCTION (Paper I)

The association between olfactory dysfunction and development of PD was first suggested in 1975 by Ansari and Johnson¹⁴³. By testing the olfactory threshold for amyl acetate in PD patients, the authors described for the first time a clear correlation between disease progression and loss of olfactory acuity. Remarkably, olfactory deficits were also reported in asymptomatic relatives of patients with PD, some of whom later developed the disease¹⁴⁴. Remarkably, most patients report a decline in their sense of smell and olfactory impairment is clinically confirmed in up to 90% of PD patients^{145–147}. Although olfactory dysfunction is one of the most frequent non-motor symptom, it remains one of the least understood¹⁴⁸.

Olfactory dysfunction is not severely disabling and it is relatively nonspecific¹⁴⁹ (up to one-third of the elderly population has olfactory loss), limiting its utility as a predictive feature of the disease. Nevertheless, given Braak's observation that the olfactory bulb is one of the first regions of the central nervous system to be affected in PD¹⁵⁰, it is important to consider this as a strategic area of research, since it may help to define features for early diagnosis (when combined with more specific prodromal symptoms).

PD is a neurodegenerative disease, but paradoxically, olfactory deficit may be a consequence of dysregulated neurogenesis.

In the adult olfactory bulb, neurogenesis continuously replaces older neurons that die and this is particularly relevant to the generation of new interneurons¹⁵¹. Interfering with this process affects olfactory discrimination^{152,153}, including odor-dependent responses, such as fine discrimination and short-term spontaneous memory of odorant cues^{154–159}. In the brain of PD patients, an increased number of dopamine interneurons is found in the glomerular layer of the main olfactory bulb (MOBgl), a feature that has been replicated in preclinical research^{160–165}. Importantly, dopaminergic MOBgl neurons play an inhibitory role (they are indeed GABAergic but capable of releasing dopamine) in the olfactory circuit¹⁴⁸ and their excessive number is believed to be responsible for the observed olfactory deficits in PD.

The mechanism of this upregulation is linked to alterations in neural progenitor cells in the subventricular zone (located in the vicinity of the striatum, surrounding the lateral ventricles), which continuously provides the olfactory bulb with newly generated cells¹⁶⁶. In the PD brain neurogenesis is reduced, yet the net effect in the MOBgl is an increase in DA interneurons. The causal links between these events are not yet fully understood.

Interestingly, olfactory dysfunction in PD is not responsive to dopaminergic replacement therapies¹⁶⁷. Thus, patients can undergo years of treatment with L-Dopa and yet continue to show olfactory dysfunction. In accord, L-Dopa treatment normalizes decreased neurogenesis in the adult brain of 6-OHDA unilaterally lesioned mice but does not correct for the increased number of dopaminergic MOBgl cells¹⁶⁸. Similarly, prolonged treatment with the non-ergoline dopamine D2 / D3 agonist pramipexole¹⁶⁹, normalizes neurogenesis in the adult brain of PD rodent models, increasing the number of newly generated neurons in the olfactory bulb (6-OHDA lesioned mice¹⁶⁸ and rats¹⁷⁰), with unknown effect on dopaminergic cell numbers within the MOBgl. The MAO-B inhibitor, selegiline, has been found to increase the number of newly generated neurons and normalizes the number of dopaminergic cells in the MOBgl (6-OHDA lesioned mice¹⁶⁷), however no studies have conclusively shown whether either of those treatments can result in olfactory recovery.

In summary, a growing body of literature is pointing to aberrant neurogenesis as the causal factor in PD olfactory deficit. Some of the drugs used for dopamine replacement therapies positively affect neurogenesis but not necessarily normalize the dopaminergic MOBgl cell numbers. These observations remain compartmented due to the lack of suitable preclinical models to examine the effect of therapeutic interventions on anatomical changes underlying olfactory performance. In order to test whether our mouse model could be used to study olfactory dysfunction in PD, we examined the olfactory discriminative ability of 6-OHDA Lesion and Sham mice.

When a mouse is exposed to a novel odor, it explores the odor by sniffing¹⁷¹. With repeated presentations of the odor, the animal spends progressively less time exploring it (habituation). Thus, we measured habituation, as well as the tendency to explore for a longer period of time a new odor (dishabituation)¹⁷² when animals were presented to different odors. We selected two categories of odors; non-social (*e.g.*, neutral) and social (that carry biological meaning, *e.g.*, used as a mean for intra-species communication). This approach allowed us to detect olfactory discriminative impairment in our PD model, consisting in the inability to recognize an odor as novel within categories (*fig.2, Paper I*). In line with these results, the olfactory deficit described in PD patients is often manifested as lack of ability to distinguish between odors¹⁴⁷.

Our observations are in line with what was described in a PD mouse model based on overexpression of human α -synuclein, which discriminates between strongly different scents, but lacks the ability to distinguish between different citrus odors¹⁷³. However, the α -synuclein overexpressing mouse model of PD develops, in parallel, a variety of symptoms constraining the timeline of studies that entail prolonged treatments, which are necessary for the study of

neurogenesis. Thereafter, our mouse model allows the experimenter to potentially observe the effect of drugs on neurogenesis and assess its effect on olfactory performance.

3.5 CIRCADIAN RHYTHM ALTERATIONS (Paper II)

Disturbances of daily pattern of sleep-wake cycle are consistently documented in PD patients¹⁷⁴. For example, many patients experience symptoms of insomnia and excessive daytime sleepiness^{175–178} which are not responsive to L-Dopa treatment¹⁷⁹. Excessive daytime sleepiness, as opposite to wakefulness (*i.e.*, alertness), has been extensively studied by Arnulf and colleagues¹⁸⁰, who suggested that circadian dysfunction is a key component of the non-motor PD symptomatology. In a recent review, David Willison¹⁸¹ described sleep disorders as nearly ubiquitous among PD patients and highlighted that those disturbances manifest early during the disease. The potential impact of a disrupted circadian system in PD has received relatively little attention in the literature yet it may be a contributing factor to the severity of other NMS.

The role of DA in the regulation of circadian clock function is now well-established. Both in non-human primates and rodent models, DA loss is associated with a disruption of sleep architecture^{182–185}, including fragmentation and onset variability of rest vs awake states^{186,187}. Concomitantly, DA is known as an important wake promoting factor across species^{188,189} and dopaminergic availability has been linked to circadian clock function with DA transmission exhibiting daily rhythms of activity^{190,191} and extracellular levels of DA affecting the patterns of striatal clock gene expression¹⁹² and neuronal excitability.

Changes in the circadian rhythm have been investigated in different animal models of PD, highlighting a correlation with DA degeneration. While MPTP-treated mice¹⁸³, which exhibit approximately 50% loss of dopaminergic neurons, do not show disruption of the circadian rhythm^{183,193}, the MitoPark mice¹⁹⁴, which have a progressive DA loss¹⁹⁵, show both motor deficits and circadian rhythm disruption at about 20 weeks of age (corresponding to ~60% loss of DA midbrain neurons). In these mice, the age-dependent decline affects the amplitude as well as the stability of the circadian rhythm, coupled with an increased fragmentation of day/night activities. If the circadian system is challenged by exposure to constant darkness, normal animals retain a robust free-running circadian locomotor rhythm, whereas MitoPark mice lose periodicity (referred to as arrhythmia). Upon re-exposure to light-dark cycle, locomotor rhythm is restored¹⁸⁶. In agreement with the results obtained in MitoPark mice, rats with a unilateral 6-OHDA lesion¹⁹¹, VMAT2-deficient mice¹⁹⁶ and the α -synuclein overexpressing¹⁹⁷ transgenic mice also show lower night-time activity (rodents are nocturnal) and in some cases fragmentation during sustained darkness¹⁹⁸.

In line with these observations, we found that in our mouse model the bilateral partial lesion disrupts the circadian rhythm and affects the spontaneous motility of the animals during the active (*i.e.*, dark) phase of the 24 h cycle, as measured by reduced movement in the activity monitoring home cage (*fig.1, Paper II*).

The light-dark cycle strongly influences the daily pattern of behavior, often over-riding the expression of underlying circadian rhythms controlled by the endogenous clock genes¹⁹⁹ (*i.e.*, entrainment). We decided to study the endogenous circadian rhythm by analyzing spontaneous motility in the free running state. This assessment requires animals to be exposed to constant conditions in absence of cyclic environmental cues, such as light²⁰⁰. We found that our mouse model displays a disruption of the endogenous circadian rhythm, as indicated by the severe fragmentation of the activity pattern observed when Lesion mice are kept in constant darkness (*fig.2, Paper II*) (know-how on analysis of rhythmicity search for Refinetti R.).

Circadian rhythm and wakefulness are regulated by histamine^{201–203}, which is widely expressed throughout the brain and participates in the regulation of basic homeostatic processes and multiple brain functions. Among the four histamine receptors, the H3 receptor (H3R) has received considerable attention as a target for the treatment of sleep disorders^{202,204–207}. H3Rs are located presynaptically, where they reduce histamine synthesis and release^{208,209}. Administration of H3R antagonists/inverse agonists, such as thioperamide, has been shown to improve wakefulness in animal models of narcolepsy and in narcoleptic patients^{207,210–212}. Thus, we tested thioperamide for its ability to revert dysfunctional circadian rhythm observed in our mouse model of PD. Our results indicate that subchronic treatment with thioperamide restores normal motor activity during the active phase of the circadian rhythm and highlights the potential efficacy of this drug for the treatment of sleepiness and reduced wakefulness in PD patients (*fig.3, Paper II*).

3.6 COGNITIVE DEFICITS (Papers II and III)

Cognition can be measured within different functional domains. For example, while attention is commonly assessed by parameters such as reaction time and vigilance, memory regards the processing of new information and is usually evaluated in recognition and executive memory tests. Other cognitive functions are very specific, like visuospatial analysis and orientation or use of language (verbal fluency and naming). When dysfunctional, all these mental capacities fall under the umbrella of cognitive deficit and are clinically classified on a growing spectrum, from mild-to-dementia, depending on how much they interfere with everyday life.

Cognitive dysfunctions, such as those listed above, are common in PD. Mild cognitive impairment affects a quarter of patients in the early stages of their disease^{213,214}, and approximately half reach dementia by 10 years from diagnosis²¹⁵. However, the pattern of cognitive impairments and their speed of evolution vary markedly between individuals²¹⁶. Some patients progress rapidly to dementia while others have slower time courses (related to age at onset of PD and strongly correlated to familial forms of the disease²¹⁷). PD patients that develop cognitive dysfunction are usually affected in at least one cognitive domain²¹⁸ prior to the emergence of motor symptoms^{219–221}.

In the preclinical research, the assessment of cognitive function is generally done in two steps. Initially, the animal is presented to stimuli and then to a novel condition in which the prior stimuli presentation serves as a guide for prospective behavior. The stimuli can belong to any sensory modality, be it visual, tactile or spatial and the novel condition can be the presence of novel cues, new contexts or locations. The prospective behavior will be dependent on natural evolutionary traits and, in the case of rodents, usually relies on their ‘unconditioned preference’ for novelty²²². Thus, when exposed to a familiar object alongside a novel object, rodents approach and spend more time exploring the novel object. This indicates that a representation of the familiar object exists in memory, allowing the rodent to attribute novelty to the new object. The commonly used **novel object recognition** (NOR) test is based on these pre-requisites. The test consists of a training phase where the animals are presented with two similar objects, and following an interval, one of the objects is replaced with a novel object, and the exploratory behavior (time spent investigating the objects) is measured²²³. The NOR test is prone to a variety of conditions under which results can be misleading. For instance, the choice of objects must be based on perceptual cues that are relevant for rodents, not humans²²⁴. The environment should not be aversive or contain unnecessary cues that could compete for attention (*i.e.*, any novel feature will result in ‘unconditioned preference’). Furthermore, the ‘cognitive load’ of this test is dependent on time (*e.g.*, interval between training and test phase).

The short nature of this test and its proven sensitivity to detect cognitive changes in our mouse model (discussed on section 1.6), encouraged us to use the same protocol in multiple occasions and proved it to be a useful method for the assessment of drug efficacy.

As discussed above, clinical and preclinical evidence indicates that the degeneration of midbrain dopaminergic neurons occurring in PD affects a number of forebrain structures, including the hippocampus²²⁵, which is critically involved in plastic processes underlying memory. In particular, novelty activates the dopaminergic mesocorticolimbic pathway²²⁶, giving rise to the hypothesis that it modulates hippocampal memory^{227,228}, through D1 receptors and transcriptional regulation of plasticity-related genes^{229–232}. Note that, although triggered by novelty, DA in the hippocampus differs from the error-detection role it serves in the ventral striatum^{233,234}.

In the initial study published by our research group, it was shown that the bilateral 6-OHDA lesion impaired long-term novel object recognition and decreased long-term potentiation specifically in the dentate gyrus of the hippocampus. These abnormalities were reverted by administration of L-Dopa, or the D1 receptor agonist SKF81297, but not by the treatment with the D2 / D3 receptor agonist, pramipexole⁶⁹.

In *Paper II* we extended this study and further characterized the effect of 6-OHDA lesion on higher brain functions, by analyzing gamma oscillations in the hippocampus. Hippocampal oscillatory activity has been associated to several cognitive functions, including long-term memory encoding and recall²³⁵, facilitation of synaptic plasticity²³⁶, and maintenance of selective attention²³⁷. Interestingly, we found that the bilateral 6-OHDA lesion disrupts

hippocampal gamma oscillations. Given the involvement of the histaminergic system in the modulation of cognitive-relevant oscillatory activity, we also examined the effect of the H3R antagonist thioperamide on hippocampal gamma oscillations. We found that thioperamide prevents the impairment of recognition memory caused by the 6-OHDA lesion and corrects the deficit in gamma oscillation. Our work proposes the involvement of disrupted gamma oscillations in PD cognitive deficit (*fig.4a&5, Paper II*).

In *Paper III* we used our mouse model to evaluate the involvement of the mammalian target of rapamycin (mTOR) signaling pathway on multiple NMS, including memory. mTOR is the key catalytic component of two large multimeric complexes, mTOR complex 1 (mTORC1) and 2 (mTORC2)²³⁸. Whereas, mTORC1 regulates a variety of cellular functions, including cell growth and proliferation, autophagy and protein synthesis, mTORC2 participates in the control of cytoskeletal dynamics and cell size.

We focused on mTORC1 signaling, because it has been shown to be required for hippocampal long-term potentiation, memory formation and consolidation^{239,240}. Two of the main downstream targets of mTORC1, the ribosomal protein S6 kinase (S6K) and the eukaryotic initiation factor 4E-binding protein (4E-BP), promote mRNA translation via activation of downstream initiation and elongation factors^{241–243}. Activation of these signaling components modulates synaptic plasticity and affects cognition through spatial and temporal coordination of protein synthesis.

Excessive activation of mTORC1 is linked to intellectual disabilities²⁴⁴, including tuberous sclerosis^{245,246} and Down syndrome²⁴⁷. Notably, the cognitive impairment observed in animal models of these two conditions is counteracted by rapamycin, a selective inhibitor of mTORC1^{245,246,248}. We found that subchronic administration of rapamycin, which effectively reduces mTORC1 activity in the brain²⁴⁹, abolishes the memory impairment produced by a partial lesion of the dopamine system. Rapamycin acts by preventing the phosphorylation of S6K and 4E-BP, which in turn regulate two parallel signaling branches implicated in the control of protein synthesis and in multiple aspects of synaptic plasticity and memory. Our results indicate that selective inhibition of S6K with PF-4708671 is sufficient to rescue memory performance (*fig.1, Paper III*).

3.7 AFFECTIVE DISORDERS

Anxiety and depression affect up to 45% of PD patients and are among the most invalidating²¹⁹ NMS. Unfortunately, L-Dopa efficacy on mood-related dysfunctions is limited and independent reports indicate that its chronic administration might even be correlated with the emergence of depression and anxiety^{250–255}.

3.7.1 Depression (Papers I and III)

The profile of depressive symptoms observed in PD patients is not identical to that reported in primary depression. Distinctive features of PD-related depression include elevated levels of dysphoria, irritability, and suicidal ideation, albeit not accompanied by increased suicide rate. Diagnosis of depression in PD is complicated by its overlap with other symptoms. For instance, lack of concentration and fatigue can also be observed in non-depressed PD patients, and symptoms such as psychomotor slowness may be independent from depression and rather result from neurological motor deficits^{256–259}. Despite increasing efforts in the study of depression in PD patients and the availability of preclinical models, PD-related mood disorders are still difficult to treat. Studies have shown that a significant percentage of patients do not respond to 1 or more trials of pharmacotherapy, suggesting inadequacy of antidepressant choice²⁶⁰. In this regard, preclinical models are critical to identify neuronal mechanisms underlying these symptoms, screen for new drug options and test for drug-drug interactions.

Depressive-like behavior and antidepressant efficacy can be studied in rodents with the **forced swim test** (FST), developed by Porsolt²⁶¹ et al. in 1977. This classic paradigm is based upon the evaluation of immobility, as a measure of ‘behavioral despair’, which rodents display when placed in a container filled with water from which they cannot escape (know-how^{262–265}). Another popular test is the **tail suspension test** (TST), which consists of a simple set-up in which mice are suspended by their tail and the time spent in immobility is measured (know-how²⁶⁶). The FST and TST show sensitivity to all major classes of pharmacological antidepressants, including desipramine, imipramine, atypical antidepressants (*e.g.*, bupropion and citalopram), and SSRIs, such as fluoxetine and paroxetine (FST^{267,268}, TST²⁶⁹). However, both the FST and the TST represent an acute situation and do not mimic the temporal features of onset, persistence, and therapeutic effects of clinical depression in patients²⁶⁸. An alternative approach is to measure anhedonia²⁷⁰, a core symptom of depressive disorder, characterized by the loss of interest in activities that are otherwise regarded as pleasurable. In rodents, anhedonia is typically measured with the **sucrose preference test**^{271,272} (though saccharin is a preferable choice). In this test animals are offered access to two water bottles, one of which contains sweetened water. Rodents naturally favor sweetened liquids as measured by preferential liquid intake.

In this thesis, we present evidence that our mouse model of PD is a valid tool for the study of depression-like phenotypes associated with PD. We employed the aforementioned behavioral tests and showed that bilateral partial DA lesion induced by 6-OHDA results in increased

immobility in the FST and TST (*fig.3, Paper I; fig.2b, Paper III*). Moreover, we also show that depressive-like behavior is observed in mice pretreated with desipramine, indicating that this condition is dopamine-related (*fig.6a, Paper I*). These findings are in line with previous work performed in the 6-OHDA-lesioned rat^{38,67,273,274}, and in other murine models of PD, such as the MPTP-treated and VMAT2-deficient mice^{196,275,276}. Furthermore, by using the sucrose preference test we found that the 6-OHDA lesion prevents the mice from developing preference for saccharin over the course of a three-day experiment, indicative of anhedonia (*fig.6*).

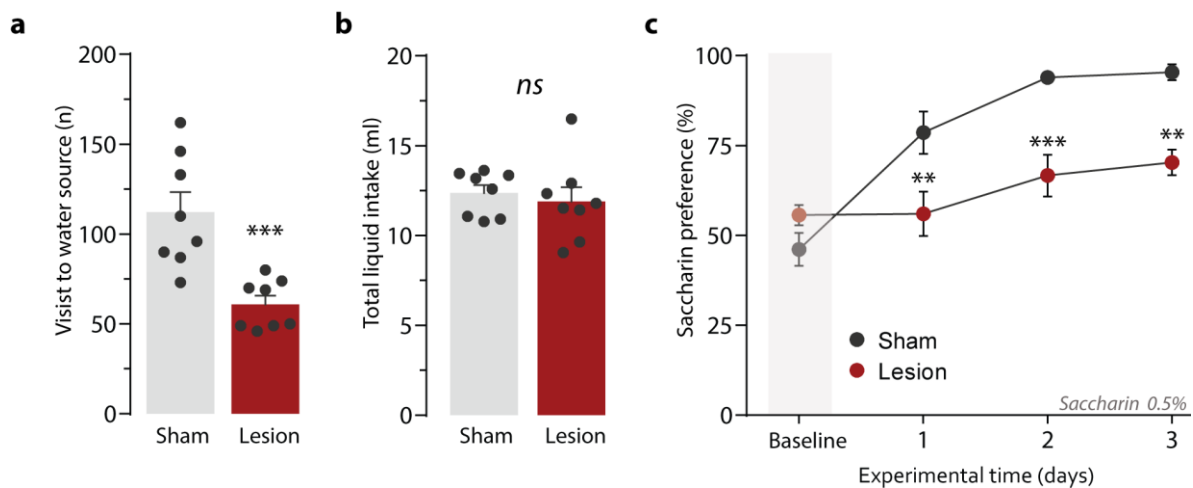


Figure 6. Measurement of anhedonia on control, sham-operated, and 6-OHDA lesioned mice (n = 8/group). Mice were first acclimatized to experimental cages for one-week (habituation) prior the experiment, and baseline preference was measured as percent (%) of volume intake from bottle 1 or 2, both filled with water (see description of the apparatus on *Paper II*). Subsequently, mice were offered 0.5% saccharin in only one of the bottles for three consecutive days. Liquid intake and visits to each bottle were measured. **(a)** Bar graph shows that over the 4 days period, Lesion mice made less visits to water source than Sham mice, in agreement with circadian data (*** $p < 0.001$, Student's t-test, two-tailed). **(b)** Bar graph shows that although Lesion mice make less visits to water bottles, both groups have similar total liquid intake (ml) ($p = 0.61$, Student's t-test, two-tailed). **(c)** Line graph shows the time course of preference for saccharin, expressed as % of saccharin intake/total intake. Note the development of preference for saccharin in Sham mice, reaching almost 100% preference within the 3 days of exposure. Whereas, Lesion mice remained close to 50% which is an indication of anhedonia (Two-way RM ANOVA, interaction effect, $F_{3,42} = 7.21$, *** $p < 0.001$, followed by Sidak's multiple comparison; between groups, ** $p < 0.01$, *** $p < 0.001$). Data presented as mean \pm SEM.

In PD, mood-related NMS appear to be dopamine-mediated. In patients, the mood fluctuations tightly correlate to the “on-off phenomenon”, with depressive symptoms increasing during the “off” L-Dopa periods²⁷⁷. Thus, unless there is a good control of the fluctuations caused by treatment with L-Dopa, antidepressant therapy will remain suboptimal²⁷⁷. In this context, drugs acting preferentially on dopamine D2 / D3 receptors, such as pramipexole, represent a potential alternative to L-Dopa, since they have been shown to reduce depression and anxiety in clinical trials^{278,279}.

Common antidepressant agents, such as noradrenaline and serotonin reuptake inhibitors, have also been tested in parkinsonian patients. For instance, tricyclic antidepressants acting on noradrenaline reuptake, such as desipramine and reboxetine, as well as citalopram, a selective serotonin reuptake inhibitor, reduce depression associated to PD^{259,280}. Similarly, nortriptyline, which blocks noradrenaline and serotonin reuptake, reduces depression in PD patients, thereby significantly improving their quality of life²⁸¹.

In agreement with clinical data, both pramipexole and reboxetine, but not L-Dopa, treatment reduced depressive-like behavior in our mouse model (*fig.5a&6a, Paper I*). Interestingly, since the noradrenaline reuptake inhibitor desipramine did not affect the depressive-like phenotype observed in our Lesion mice, it is unlikely that reboxetine produces this effect by promoting noradrenergic transmission. A more plausible explanation is that reboxetine acts by blocking dopamine uptake from noradrenaline terminals, which is particularly efficient in extra-striatal regions involved in affective functions, including the cerebral cortex and hippocampus^{73,282}.

Dysregulated mTOR transmission (discussed on section 3.6) is also implicated in affective disorders. A current hypothesis is that decreased mTORC1 activity in different cortical regions is associated with depression whereas augmented mTORC1 activity, such as that produced by the NMDA receptor agonist ketamine, reverts these conditions^{283–286}. However, studies in animal models have shown that subchronic administration of rapamycin reduces depressive-like behaviors²⁸⁷, prompting further analysis of the actions of this drug on mood disorders. In *Paper III* we used our mouse model to examine the ability of rapamycin and PF-4708671, two inhibitors of mTORC1 signaling, to counteract depressive-like behaviors associated with PD. We found that only rapamycin reduces the depression-like behavior manifested by PD mice in the FST, suggesting that the anti-depressant action of rapamycin depends on concomitant inhibition of the 4E-BP and S6K signaling cascades. The finding that rapamycin reduces depression-like behavior contrasts with previous studies indicating that reduced mTOR signaling is associated with depression. In this regard, our results are more in line with the observation that subchronic administration of rapamycin, albeit at higher doses²⁸⁷ than those used in our study, exerts anti-depressant effects in the FST and TST (*fig.2, Paper III*).

3.7.2 Anxiety (Papers I, II and III)

In nature, anxiety is an adaptive response and serves as an alerting signal of impending danger, finalized to adjust the behavior and implement appropriate countermeasures²⁸⁸. However, anxiety may also be pathological if the symptoms are prolonged, excessive or occurring at inappropriate times. Anxiety and depression are sometimes difficult to distinguish; however, unlike depression, a core feature of anxiety is the presence of apprehension and worry.

There is increasing evidence that anxiety disorders in PD may be directly related to early neuropathological changes^{289–291} and despite their high prevalence, anxiety disorders are often

under-diagnosed and under-treated in PD patients²⁹². In the clinics, anxiety disorders often precede or are accompanied by depression (comorbidity) and, in many cases, may persist even if depression is successfully treated²⁹³.

In preclinical research, anxiety can be measured through the expression of defensive behaviors (*i.e.*, actions the animal takes to reduce threat). Defensive behaviors vary according to species and are dependent on environmental features. Distinctions between the expression of anxiety and fear are much debated in the literature. For example, when rodents are enclosed on a small environment and exposed to a threat they show a freezing response, interpreted as fear, but when an “escape” route is available, rodents exhibit anxiety-induced avoidance behavior²⁹⁴. Thereafter, behavioral tests for fear or anxiety must take into consideration the environmental features and in particular the availability of an “escape” route.

The most widely used and pharmacologically validated procedures for assessing anxiety in rodents are the **open field** (OF) and the **elevated plus maze** (EPM) tests (know-how^{295–297}). The OF assesses anxiety-like behavior by measuring the extent to which the animal avoids the center of the arena, which entails risk, and stays close to the walls, a behavior known as thigmotaxis. In the EPM rodents are placed on an elevated cross-shaped maze with two open arms and two enclosed arms. Rodents naturally avoid the open arms, which entail risk, and excessive avoidance is an indicator of anxiety. Anxiety can also be assessed using the **light-dark box** test (know-how²⁹⁹). Rodents have innate aversion to brightly illuminated areas and the light-dark apparatus consists of a dark “safe” chamber with an open access to an illuminated “aversive” chamber. Rodents are initially placed in a dark compartment and place avoidance is measured as the latency to visit the brightly lit area^{298–300}.

We have tested the PD mouse model with the three tests presented above and our results showed that 6-OHDA lesion increases thigmotaxis in the OF, reduces exploration of the open arms in the EPM and increases the latency to the first exploration of the bright chamber in the light-dark box test. These responses are indicative of increased anxiety (*fig.4, Paper I; fig.4b&c, Paper II; fig.3&4, Paper III*). Interestingly, pretreatment with desipramine did not modify the anxiogenic effect caused by the 6-OHDA lesion (*fig.6b, Paper I*), suggesting that the loss of nigrostriatal dopaminergic neurons specifically mediates mood-related disorders in our model of experimental parkinsonism²⁷⁴.

In summary, we validated our 6-OHDA mouse model of PD for the study of depression and anxiety, behaviors observed in PD patients^{301–303}. Though few publications have shown depression and anxiety features in the same PD model (comorbidity), a variety of dopamine depleted rodents do manifest increased anxiety-like behaviors^{67,196,304,305}. Tadaiesky et al. (discussed on section 1.5) suggested that the anxiogenic response elicited by 6-OHDA could be associated with dopaminergic depletion in the prefrontal cortex. This hypothesis is supported by previous evidence that 6-OHDA injected directly into the medial prefrontal cortex induces a significant anxiogenic effect in rats evaluated in the EPM³⁰⁶.

In contrast with the idea of a pivotal role of the prefrontal cortex in PD related anxiety, functional magnetic resonance imaging studies suggest that abnormal emotional responses in PD patients might be mediated by perturbed dopaminergic input to the amygdala^{307,308}. This brain area plays a fundamental role in the acquisition, modulation and expression of emotions, being targeted by dopaminergic cells and having wide-spread efferent connections to nuclei within the basal ganglia^{309,310}. In the amygdaloid complex, dopamine originates from the substantia nigra and ventral tegmental area, which target the main input (basolateral, BLA) and output (central, CeA) nuclei of the amygdala (for review³¹¹). Interestingly, brain wide analyses of TH expression in our mouse model indicate that the dopaminergic denervation caused by the 6-OHDA lesion is more prominent in the BLA (*data not shown*, approach presented on section 3.2). Remarkably, it has been shown that whereas the BLA is involved in stimulus detection and evaluation of the threatening stimuli linked to anxiety, the CeA is involved in the expression of fear behaviors³¹².

Our limited understanding of the underlying pathophysiological mechanisms that give rise to anxiety is evidenced in the extensive literature containing conceivable factors that could explain mood disorders in PD.

Recent evidence suggests that the noradrenergic and serotonergic systems may play a more relevant role in the manifestation of PD-related anxiety than previously thought²⁵⁴. According to the Braak staging of PD pathology, serotonergic cell loss in the raphe nuclei is evident prior to nigrostriatal dopaminergic degeneration^{19,313}. Serotonergic neurons originating in the raphe nuclei provide a massive input to corticolimbic structures involved in the control of anxious states, and there are many studies demonstrating that anxiety disorders may be caused by serotonin abnormalities²⁸⁸.

Noradrenaline dysfunction also occurs prior to pronounced degeneration of dopaminergic neurons¹⁵⁰. Ascending noradrenergic projections from the locus coeruleus heavily innervate essentially all corticolimbic regions involved in integrating the response to anxiety. Due to noradrenergic cell loss in the locus coeruleus of PD patients¹⁹, significant changes in the expression of noradrenaline receptors and transporters may cause the development or exacerbation of anxiety. Furthermore, lower levels of dopamine and noradrenaline transporters in the locus coeruleus are correlated with increased incidence of anxiety in PD patients³¹⁴. While plasma noradrenaline levels are elevated in *de novo* PD patients³¹⁵, lower levels of dopamine beta-hydroxylase, the enzyme responsible for hydroxylation of dopamine to noradrenaline, have been observed in the cerebrospinal fluid of L-Dopa-treated PD patients^{316,317} suggesting that L-Dopa treatment may alter noradrenaline levels.

In *Paper I*, we showed that the administration of the dopamine precursor L-Dopa did not modify anxiety-like behaviors in our mice, whereas the D2 / D3 receptor agonist, pramipexole, efficiently reduced anxiety features in the EPM (*fig.5b, Paper I*). A similar effect was observed following reboxetine treatment, likely mediated by increasing DA availability in extra-striatal regions since desipramine pre-treatment did not block the anxiogenic effect caused by the lesion (*fig.6b, Paper I*).

In *Paper II* we tested thioperamide, a drug that corrected circadian dysfunction and exerted pro-cognitive effects in our PD mouse model. We pretreated mice with thioperamide before the EPM and light-dark box test based on Bahi et al. previously reported evidence that administration of an H3R antagonists, which promotes histamine release, counteracts anxiety in naïve mice³¹⁸. However, thioperamide did not affect the anxious phenotype produced by partial dopamine depletion (*fig.4b&c, Paper II*). This highlights the importance of utilizing appropriate animal models of disease when assessing the efficacy of therapeutic interventions for specific pathological conditions.

Finally, in *Paper III* we tested the effect of the mTORC1 inhibitor rapamycin and showed that subchronic treatment counteracts the anxiety-like behavior by normalizing the time spent by PD mice in the center zone of the OF, thereby reducing thigmotaxis. We also showed that rapamycin increases the propensity of PD mice to explore the open arm of the EPM apparatus. In parallel experiments, we used the selective S6K inhibitor, PF-4708671, which did not produce a reduction of anxiety-like behaviors (*fig.3&4, Paper III*). These results indicate that rapamycin is capable of fully rescuing anxiety-like behavior in a mouse model of PD, and that this effect likely requires blockade of multiple downstream targets of mTORC1.

4 CONCLUSION AND FUTURE PERSPECTIVES

The diagnosis and, in large part, the treatment of PD are still focused on motor deficits. This criterion has likely contributed to the dominance of motor-based animal models for the study of this disorder. However, as shown throughout this thesis, the emerging picture of PD is that of a multi-system disorder with motor and non-motor features. NMS in PD are highly prevalent and include olfactory loss, sleep and circadian rhythm disturbances, cognitive deficits, depression and anxiety, among others. While some of these symptoms respond to dopaminergic therapies, others are treatment-resistant and represent the current challenge faced in the management of PD symptomatology.

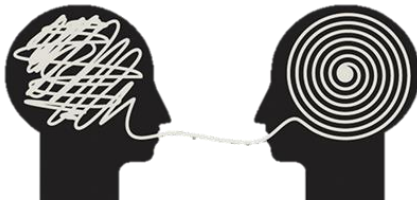
The unmet need for treatments able to counteract NMS highlights the importance of shifting the attention from motor models of PD to NMS models. Although PD is a human-specific disease, and does not occur spontaneously in animals, some features of PD can be induced in laboratory animals through the administration of toxins, such as 6-OHDA. In this thesis, we validated a 6-OHDA-mouse model reproducing a wide range of NMS and used it to identify new potential therapeutic approaches for PD.

Our findings shed light on the contribution of the dopamine and noradrenaline systems to affective symptoms frequently manifested in PD patients, indicating the prominent role of dopaminergic transmission. We also identified histamine H3 receptor antagonists as potential drugs for the treatment of circadian rhythm disruption, which is commonly observed in PD. Notably, using our model we showed that administration of drugs interfering with the mTOR signaling cascade reverts a number of psychiatric symptoms associated with PD. In particular, we found that rapamycin, a drug approved for use as an anti-tumoral, reverts the memory deficit, as well as depression- and anxiety-like behaviors produced by the 6-OHDA lesion.

The model described in this thesis represents a relatively simple tool to identify specific pharmacological approaches for the treatment of NMS. We have shown that a number of NMS can be reproduced in this model and we intend to extend this characterization. For instance, it will be interesting to test whether the 6-OHDA lesion results in modifications of oscillatory activity at cortical and hippocampal level responsible for sleep alterations, such as REM sleep behavior disorder. These studies will be particularly important, since oscillatory activity during sleep has been proposed to play an important role in memory consolidation³¹⁹.

Other studies will be needed to further investigate the use of specific drugs for the treatment of psychiatric symptoms affecting PD patients. The finding that rapamycin counteracts cognitive and affective deficits points at a large class of substances acting on mTOR and related signaling pathways as potential drugs against NMS. Importantly, many of these compounds have already been approved for clinical use in other pathologies, thereby prompting the analysis of their possible repositioning for the treatment of PD.

5 'Plain Language' summary



Parkinson's disease (PD) is brought on by the degeneration and death of the neurons in the part of the brain that produces dopamine. It's typically discovered with the onset of motor symptoms that include hand tremors and body stiffness. Clinicians have no final diagnostic test to rely on, so they typically look

for motor symptoms and then administer medicine to see if they will dissipate. If a patient responds to the treatment, that means he/she is very likely to have PD.

The medicines used to treat these motor symptoms were introduced in the 1960s, and they all follow one logic. They aim to replenish dopamine since it is reduced in the brain. There's no cure for PD, and these therapies simply control symptoms. Over the course of decades researchers worked hard to figure out the best drug cocktail and the more they learnt, the more nuances they saw. For example, researchers noticed that people with PD suffer from a variety of other conditions which are not strictly motor. They have problems sleeping and feel tired all day long, almost all of them lose their sense of smell, some have mild dementia and nearly half get depressed or anxious. These and many other conditions are what we call non-motor symptoms, they occur in PD patients and truly affect their quality of life. The aim of this thesis was to provide scientists with a tool to study non-motor symptoms in PD. Such a tool should have as many of the non-motor symptoms as possible and those should occur due to a similar cause, meaning that neurodegeneration and lack of dopamine were mandatory features.

Studying PD is difficult because only humans have this condition. Moreover, the workings of the brain are very complex and largely mysterious. We had to choose a living organism, and this animal should have a complex brain, comparable to our own. We selected the mouse. Mice in research have made incredible contributions to medicine because they are in so many ways comparable to us (see ethical considerations on *p.11*).

We used a toxin called 6-OHDA, this toxin kills the brain cells that produce dopamine and, we used it to recreate the mandatory features described above. We carefully made sure to inject precise amounts of the toxin into the brain in order to get an animal that would have the non-motor symptoms yet be motorically healthy. This was important because animals cannot report how they feel. Hence, we need to 'read' their body-language and for that we rely on movement.

We showed that our mouse model of PD partially lost its sense of smell, that it behaved as if 'it was tired' all day long, that it had learning issues and even depressive and anxiety features. Thus, we believe this model allows for the study of non-motor symptoms in PD.

We then used our PD mice to learn why certain medicine help and others don't. Finally, we searched for alternative treatments. We selected drugs that are approved for use in the clinics but not currently used for the treatment of PD. This is called 'repositioning' and this kind of approach cannot be directly used in humans because it risks causing harm. By repositioning, we found a new category of drugs that could be used for the treatment of non-motor symptoms in PD.

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At first, I thought science was a simple scheme. In point A, there is a question, and one proceeds by the shortest path to point B, where the answer lies. You educated me by showing that enclosed mental schemes pose a danger because any deviation can bring undue stress. I learnt from you that the path is a ‘cloud’, and that in the midst of confusion one can better sense the problem and resources at hand. From the process, a novel approach unfolds. I learnt from you that one does not move ‘to B’, but rather ‘towards B’.

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